Silybum marianum in vitro-flavonolignan production

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ABSTRACT

The effect of coniferyl alcohol as a precursor of flavonolignan biosynthesis on silymarin components production in *Silybum marianum* suspension culture was studied. Coniferyl alcohol showed the changes in silymarin complex production. Silydianin was detected mainly in the control samples of cultivated cells. A significant increase of silydianin was observed only after 72 h of the application of 46μM coniferyl alcohol. No other components of the silymarin complex (silychristin and silybin) were detected; neither in control samples nor after the precursor feeding. But the increased accumulation of taxifolin (flavanole) was very interesting. The highest taxifolin production was reached after 48 hours of treatment (about 554% compared to control).

Keywords: milk thistle; *Silybum marianum*; *in vitro* culture; flavonolignans; coniferyl alcohol

Milk thistle, *Silybum marianum* (L.) Gaertn., Asteraceae is widely used for hepatic and biliary disorders in Europe; it has been used medicinally since the first century. It was also mentioned in the writings of Dioscorides, Jacobus Theodorus and Culpepper. Its leaves, flowers and roots were historically considered a vegetable in European diets, and fruits (achenes), which resemble seeds, were roasted as a coffee bean substitute. The leaves of the plant are eaten in fresh salads and as a spinach substitute, the stalks like asparagus, and the flower heads serve as artichokes (Luper 1998).

The milk thistle is an annual herb or biannual herbaceous plant that is widespread in temperate climate of the American countries, Australia, Southern and Western Europe. This plant is also cultivated in the Czech Republic.

The dried seeds contain 1–4% of silymarin flavonoids. Silymarin is a mixture of three flavonolignans, including silybin (silibinin), silidianin, and silichrystin (Figure 1). Other flavonolignans identified in *S. marianum* include dehydrosilybin, deoxysilycistin, deoxysilydianin, silandrin, silybinome, silyhermin, and neosilyhermin. In addition, milk thistle contains apigenin, taxifolin, silybonol, myristic, oleic, palmitin, and stearin acids (Wichtl 1994).

The parts of silymarin complex are of a considerable pharmacological interest owing to their strong anti-hepatotoxic and hepatoprotective activity (Valenzuela et al. 1986, Morazoni and Bombardelli 1995), and a recent work has shown that silymarin also acts as an anticholesterolaemic agent (Krecman et al. 1998). Milk thistle fruit is used for the prophylaxis and treatment of liver damage caused by metabolite toxins, e.g. alcohol, tissue poison, in liver dysfunction and after hepatitis (post-hepatic syndrome), and in chronic degenerative liver conditions (Wichtl 1994).

In vitro culture of cells and tissues may offer an alternative for the production of silymarin but until now, only few studies addressing this possibility have been carried out (Becker and Schrall 1977), and in all cases flavonolignan production in *in vitro* cultures is very low and even disappears in prolonged cultures. This is a common occurrence in the production of secondary metabolites in many species. However, in some cases (Corchete et al. 1991, Hagendoorn et al. 1994), the manipulation of the components of the culture medium substantially increased the production of these compounds.

Feeding experiments with the precursors of biosynthesis of secondary metabolites resulted

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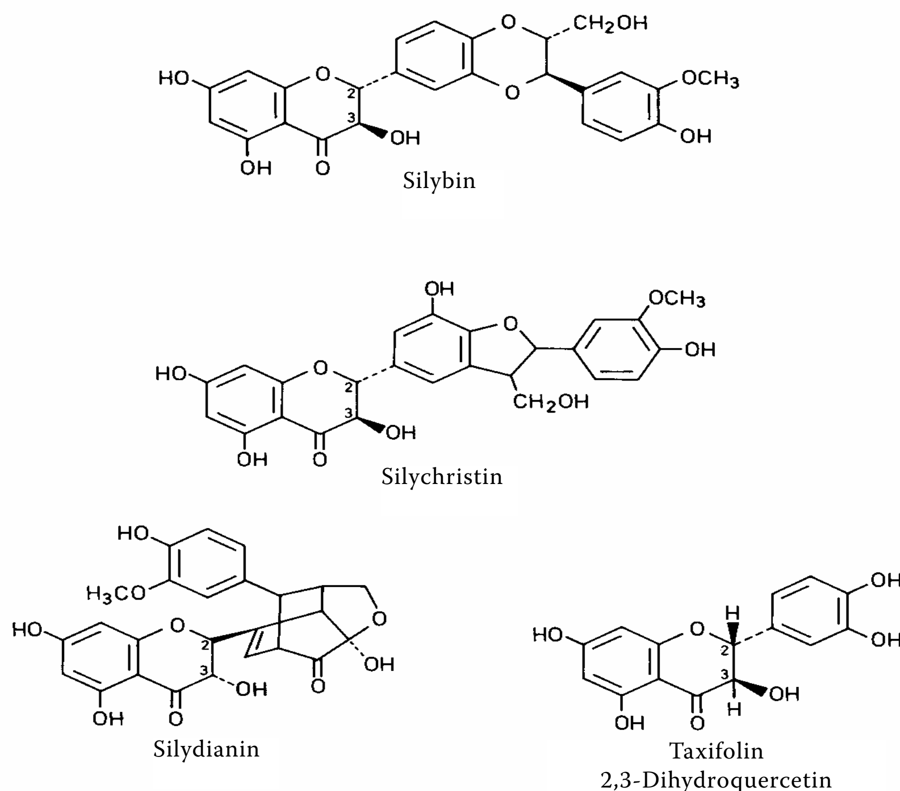

Figure 1. Main components of silymarin complex and taxifolin structure

in a 3–5 fold increase in levels of secondary metabolites. For example precursor L-phenylalanine showed a 3–5 fold increase in 5-methoxypodophyllotoxin in suspension culture of *Linum flavum* (van Uden et al. 1990).

Coniferyl alcohol is a key precursor of biosynthetic pathway of flavonolignans. Flavonolignans are adducts of flavanole taxifolin with coniferyl alcohol (Figure 2). Coniferyl alcohol was chosen as a model substance in this study. The aim of our experiment was to increase flavonolignan production by feeding nutrient medium with precursor of coniferyl alcohol. The same precursor – coniferyl alcohol in the form of complex with β-cyclodextrin was used as precursor for podophyllotoxin accumulation in *Podophyllum hexandrum* cell suspension cultures. The β-cyclodextrin itself was proven to be non-toxic for cells. It did not influence podophyllotoxin content and it was not metabolized or used as a carbon source by cells. For comparison, coniferin, the water-soluble β-D-glucoside of coniferyl alcohol, was also fed in the same concentrations. The effect of coniferin on the podophyllotoxin accumulation was stronger than that of coniferyl alcohol complexed with β-cyclodextrin, but coniferin is not commercially available (Woerdenbag et al. 1990).

MATERIAL AND METHODS

Plant material. Cell suspensions were established from 3-month-old undifferentiated cotyledon calluses and grown in MS liquid medium (Murashige and Skoog 1962) supplemented with 10 mg/dm^3 NAA. These suspensions were cultivated in growth chambers at 25°C in the shaker at 120 rpm and 16 hours daylight. Ethanolic coniferyl alcohol solution in concentrations c_1 33.11 mg/dm^3 (184μM); c_2 16.56 mg/dm^3 (92μM) and c_3 8.28 mg/dm^3 (46μM) was added into MS nutrient medium as biosynthesis precursor of flavonolignans. After 12, 24, 48, 72, and 168 hours of precursor application, the suspension cells were taken out. The cells were separated from nutrient medium by a reduced press filtration and were dried at the laboratory temperature. The flavonolignan content was detected not only in suspension cells but also in the nutrient medium.

Analytical method (Řimáková 2005). Dried suspension cells were extracted in methanol for 30 min and methanolic solution was concentrated to 5 ml and analyzed by HPLC. Solid fraction after evaporation of nutrient medium was dissolved in 5 ml of methanol. The content of flavonolignans was determined by HPLC on a UNICAM CRYSTAL

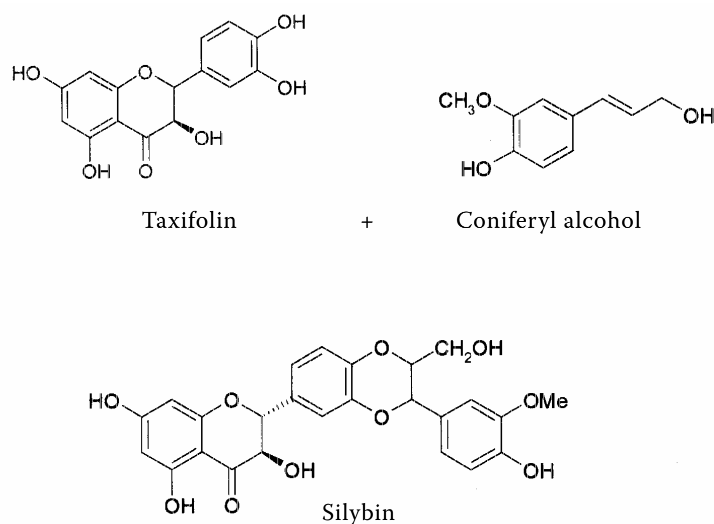

Figure 2. Biosynthesis of flavonolignans

200 Liquid Chromatograph, column LiChrospher RP-18 (250 mm × 4 mm). HPLC conditions were developed in our laboratory as follows. The mobile phase consisted of methanol and water (both acidified with 0.3% orthophosphoric acid p.a. – w/v). Flavonolignans were eluted with linear gradient from water to 50% methanol in 5 min, following by isocratic elution with 50% methanol for 20 min. The flow-rate was 1.4 ml/min. Substances were detected by absorption at λ = 288 nm and their identification was carried out by the comparison of retention times and absorption spectra with standard complex of silymarin (Sigma-Aldrich). All experimental analyses were carried out in a minimum of three independent complexes for each time and each concentration of precursor. The content of flavonolignans was referred to the dry mass of suspension complex. Statistical significance was calculated using Student's *t*-test for unpaired data ($P \leq 0.05$).

RESULTS AND DISCUSSION

The feeding of precursors to differentiated cell cultures, resembling organised tissue of intact plant, may open new ways for the improvement of secondary metabolites. Cell culture of *Taxus cuspidata* represents an alternative to whole plant extraction as a source of taxol and related taxanes. Feeding phenylalanine to callus culture was previously shown to result in increased taxol yields, probably due to the involvement of this aminoacid as a precursor or the N-benzoylphenylisoserine side chain of taxol. Also all other tested precursors (benzoic acid, N-benzoylglycine, serine, glycine) increased taxol accumulation. The greatest increases in taxol accumulation were observed in the presence of various concentrations of phenylalanine, and benzoic acid (Fett-Neto et al. 2004).

Our experiments with coniferyl alcohol as a precursor used in suspension culture of S*ilybum marianum* showed the changes in silymarin complex production. Silydianin was detected mainly in the control samples of cultivated cells. A significant increase of silydianin was observed only after 72 h of the application of 46μM coniferyl alcohol. No other components of the silymarin complex (silychristin and silybin) were detected; neither in control samples nor after the precursor feeding. But the increased accumulation of taxifolin (flavanole) was a very interesting phenomenon. A significant increase of taxifolin content in nutrient medium (about 170%; 554%; 343% and 320%) was observed after 24; 48; 72 and 168 hours of treatment with 184μM of coniferyl alcohol (Figure 3). The greatest taxifolin production was reached after 48 hours (about 554% compared to control). High taxifolin production was detected also after 72 and 168 hours (about 118% and 192%, respectively) when coniferyl alcohol in concentration of 92μM was used (Figure 3). These results were achieved in comparison with the precursor-feeding experiments in podophyllotoxin production. Feeding with 3mM coniferyl alcohol dissolved in the culture medium as a β-CD complex, resulted in an enhanced podophyllotoxin accumulation, with a maximum of 0.012% on day 10 of the growth cycle. Non-complexed coniferyl alcohol, suspended in the medium in a concentration of 3mM, enhanced the

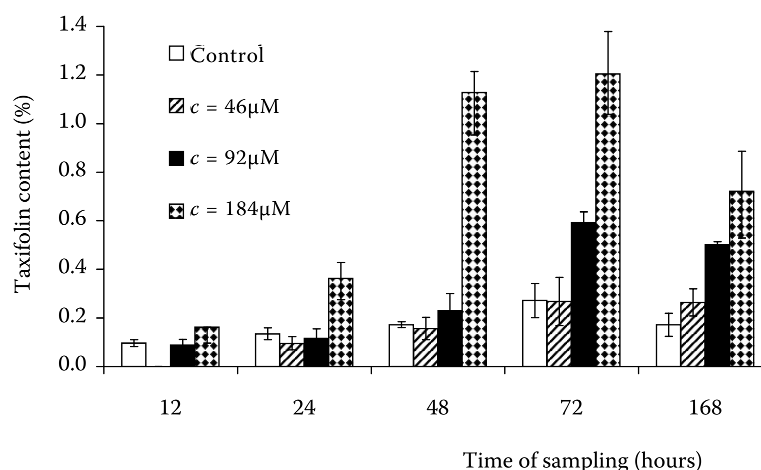

Figure 3. Taxifolin content in the medium after the treatment of *Silybum marianum* suspension culture with coniferyl alcohol

podophyllotoxin production, but only to a maximum of 0.006%. Feeding with 3mM coniferin caused a giant increase, with the maximum of 0.056% on day 10 (Woerdenbag et al. 1990).

Increased silydianin production after coniferyl alcohol feeding (as a biosynthesis precursor) was also achieved but other components of silymarin complex were not influenced. It could be caused by the use of free coniferyl alcohol only, not in the form with complex of β-cyclodextrin for better water solubility. Some precursors are very poorly soluble in aqueous media and reaction rates are therefore too low to be detected. So far, poorly water-soluble precursors have often been applied in two-phase systems. However, many of the plant cells used hardly converted precursors in the presence of organic phases, often due to dramatic decrease of cell vitality (Beiderbeck and Knoop 1988).

From the results presented, it is concluded that feeding a medium with precursor of coniferyl alcohol occurring in the lignan biosynthesis offers the possibility to enhance the content of some components of silymarin complex and flavonoid-taxifolin in *Silybum marianum* cultures *in vitro*.

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